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NITROXYL ANALOGS AS INHIBITORS OF ALDEHYDE DEHYDROGENASE

C-NITROSO COMPOUNDS

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Abstract—We previously postulated that the catalase-mediated oxidation of cyanamide leads to the formation of the unstable intermediate, *N*-hydroxycyanamide, which spontaneously decomposes to nitroxyl, the putative inhibitor of aldehyde dehydrogenase (EC 1.2.1.3; AlDH). Since it was not possible to provide direct evidence for the inhibition of AlDH by nitroxyl, we examined the activity of three representative *substituted* nitroxyls (C-nitroso compounds), viz. nitrosobenzene (NB), 1-nitroso-adamantane (NA), and 2-methyl-2-nitrosopropane (MNP), as direct inhibitors of yeast AlDH *in vitro*. While NB and NA were highly effective inhibitors in this system exhibiting IC_{50} values of 2.5 and 8.6 μM, respectively, MNP was considerably less effective with an IC_{50} of 0.15 mM. When tested *in vivo*, NA did not show any inhibitory activity on the hepatic AlDH, possibly due to the lack of site-specific delivery of the active monomeric form of this compound. However, NB at a low dose did inhibit hepatic AlDH as reflected by an increase in blood acetaldehyde levels. These results attest to the abilities of NB and NA to act as direct inhibitors of AlDH analogous to nitroxyl itself.

The mechanism of the catalase-mediated bioactivation of the alcohol deterrent agent cyanamide has been postulated to proceed via *N*-hydroxycyanamide, an unstable intermediate that decomposes to cyanide and nitroxyl (HN=O§, nitrosyl hydride), the latter the putative inhibitor of aldehyde dehydrogenase (AlDH) (Equation 1) [1, 2].

As nitroxyl is unstable and rapidly dimerizes to hyponitrous acid, which, in turn, dehydrates to nitrous oxide (N_2O) and H_2O (Equation 2), the evidence for the formation of nitroxyl from cyanamide has been indirect.

For example, the N_2O formed as the end-product of cyanamide bioactivation was shown to be derived from the amino nitrogen of cyanamide by tracer studies with the ^{15}N-labeled compound [2].

Although direct proof for the inhibition of AlDH by nitroxyl is difficult to provide and indeed may not be possible due to the highly unstable nature of this species, *substituted* nitroxyls represented by the general formula, RN=O, viz. C-nitroso compounds, where R is an aryl group or an alkyl group lacking an α-hydrogen (since a nitroso group on a primary or secondary carbon in the aliphatic series would exist in the more stable tautomeric oxime form), may represent relatively stable analogs of nitroxyl which could be tested as *direct* inhibitors of AlDH. Such C-nitroso compounds can theoretically be produced *in vivo* by oxidative metabolism of the corresponding amines via the intermediate hydroxylamines, or be generated as intermediates themselves during the enzymatic reduction of the corresponding nitro compounds to the amines. Ample precedents exist especially for the aromatic series that these C-nitroso compounds represent metabolic intermediates derived from their respective amines or nitro compounds. However, except for the benzene series itself, C-nitroso compounds of the polycyclic aromatic hydrocarbon series are mutagenic substances [3], thereby precluding their clinical use as potential alcohol deterrent agents.

In contrast, there is a paucity of biological data on C-nitroso compounds in the aliphatic series. Their chemical description is also extremely limited because of the requirement that the nitroso group be attached to a tertiary carbon. Aliphatic C-nitroso compounds have been reported to be produced *in vitro* from the corresponding amines on incubation of the latter in air with hepatic microsomes in the presence of an NADPH-generating system [4, 5]. Moreover, 1-nitrosoadamantane (see Fig. 1) was found to be excreted in the urine of rabbits after administration of the antiviral and anti-parkinsonism drug amantadine (1-aminoadamantane) [5]. In the presence of an NADPH-generating system under anaerobic conditions, NA can also be reduced to *N*-hydroxy-1-aminoadamantane (1-adamantylhydroxylamine) by a C-nitroso reductase in the microsomal fraction of liver, lung, intestine, and kidneys of experimental animals (rabbit, hamster, guinea pig, mouse, rat), with rabbit liver microsomes having the

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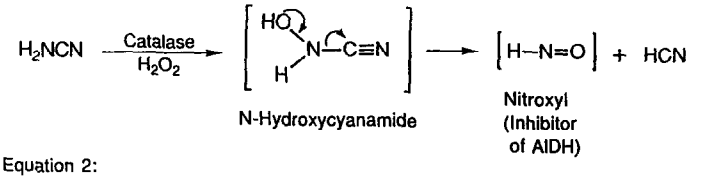
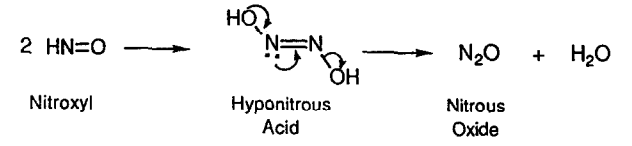
§ Abbreviations: AcH, acetaldehyde; AlDH, aldehyde dehyrogenase; CMC, carboxymethylcellulose; CP, chlorpropamide; DMSO, dimethyl sulfoxide; MNP, 2-methyl-2-nitrosopropane; NA, 1-nitrosoadamantane; NB, nitrosobenzene; $CHCl_3$, chloroform; HN=O, nitroxyl, nitrosyl hydride; and N_2O, nitrous oxide.

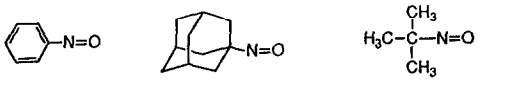
Equation 1:



Equation 2:



Equation 3:



Nitrosobenzene (NB) | 1-Nitrosoadamantane (NA) | 2-Methyl-2-nitrosopropane (MNP)

Fig. 1. Chemical structures of C-nitroso compounds.

highest activity [6, 7]. Aliphatic and aromatic C-nitroso compounds also bind to cytochrome P450 in the reduced Fe(II) state to give a characteristic Soret band at 455 nm [8–10]. As with nitroxyl itself, C-nitroso compounds tend to dimerize readily and to exist as dimers in the solid state (Equation 3). Solution in polar, preferably aprotic, solvents is required for the dimers to dissociate to the monomeric forms, thereby imparting a characteristic blue color to the solution [11].

We herewith present evidence that three representative C-nitroso compounds, viz. nitrosobenzene (NB), 1-nitrosoadamantane (NA), and 2-methyl-2-nitrosopropane (MNP) (Fig. 1), do not require metabolic activation and are direct inhibitors of yeast AlDH *in vitro*.

METHODS

Chemicals. NB was purchased from the Aldrich Chemical Co. (Milwaukee, WI), while MNP was prepared by oxidizing *N-tert*-butylhydroxylamine with diethyl azodicarboxylate, a reagent used to oxidize aromatic hydroxylamines to the corresponding nitroso compounds [12, 13], as described below.

Preparation of MNP. To a cooled (ice bath), stirred solution of 3.50 g (39.4 mmol) of *N-tert*-butylhydroxylamine [14] in 15 mL of tetrahydrofuran was added 6.86 g (39.4 mmol) of diethyl azodicarboxylate (Aldrich Chemical Co.), and the reaction allowed to proceed at 0° for 30 min and at room temperature for an additional 30 min. The precipitate of reduced reagent that formed was removed by filtration and the filtrate was diluted with 60 mL of cold water and stirred for 2 hr. The crude MNP which adhered to the walls of the flask was separated by decantation of the liquid phase and suspended in 10 mL of isooctane. The mixture was then distilled using a short condenser. The distillate boiling between 60 and 72°, which contained the monomeric product (blue) plus some entrained solvent, was collected. The blue monomer was converted to the colorless dimer of MNP and solidified on cooling, 1.90 g (56% yield), m.p. sublimes at 80° (reported m.p. 66–67° or 79–80° [11]).

Preparation of NA. This compound (dimer) was prepared by oxidation of 1-adamantylhydroxylamine following a published procedure [5]; it was recrystallized from benzene–methanol, m.p. >105° to a blue melt (monomer), i.r. spectrum (KBr) 1525 cm^{-1} (reported m.p. 103–105° to a blue melt; i.r. spectrum [nujol] 1560 cm^{-1} [5]). Its elemental analysis (C,H,N) was within ± 0.4% of the theoretical values. A colorless solution of the dimer in $CHCl_3$, λ_{max} 300 nm (ε 7000) monomerized to the blue monomer at room temperature with a concomitant increase in absorption at 690 nm (first-order rate constant, 1.0×10^{-3} sec^{-1}, 23°). Corroborating this, when NA was first dissolved in $CHCl_3$, TLC (fluorescent silica gel plates) showed two fluorescent quenching spots, R_f 0.60 (dimer) and 0.90 (monomer). After 30 min in solution, the spot for the dimer disappeared and only the monomer spot was present on TLC.

Inhibition of yeast AlDH in vitro *by NB, NA and MNP.* Yeast AlDH (0.1 IU) was preincubated for 10 min (or at various times for the time course) at

37° in a primary reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5) and/or inhibitor dissolved in the solvent designated in the figure legends, in a total volume of 0.1 mL. At 10 min, a 20-μL aliquot of the primary mixture was added to a cuvette containing 0.5 mM NAD^+, 1.0 mM EDTA, 30% glycerol and 90 mM potassium phosphate buffer (pH 8.0) in a final volume of 1.0 mL. This secondary reaction was initiated by the addition of benzaldehyde (0.6 μmol). Yeast AlDH activity was determined spectrophotometrically at 25° by following the increase in absorbance at 340 nm over time.

Animals and drug administration protocols. These studies were performed in adherence with guidelines established in the *Guide for the Care and Use of Laboratory Animals* published by the U.S. Department of Health and Human Resources (NIH Publication 85-23, revised 1985). The animals were housed in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC), and the research protocol was approved by the Animal Studies Committee of the Minneapolis VA Medical Center.

Male rats (Sprague–Dawley descent, Bio-Labs, Inc., St. Paul, MN) weighing between 160 and 220 g were fasted for a minimum of 9 hr and the drugs were administered (1.0 or 0.5 mmol/kg, i.p.) as suspensions in aqueous carboxymethylcellulose (CMC) or solutions in peanut oil (see Table 1). Two hours later, the animals were given ethanol [2.0 g/kg, i.p., as a 20% (w/v) aqueous solution]. The animals were killed [15] 1 hr later for the measurement of blood acetaldehyde (AcH, *vide infra*). Control groups received vehicle alone followed by ethanol 2 hr later.

Dosage forms of NB, chlorpropamide (CP) and the dimer of NA were prepared by suspending the material (finely ground for solids) in 2% aqueous CMC solution. For peanut oil as the vehicle, the dimer of NA was dissolved in methylene chloride and allowed to stand for 16 hr at room temperature to completely dissociate to the blue monomer. The solution was then diluted with peanut oil and placed in a rotating evaporator using water aspiration to remove the methylene chloride until constant weight was achieved. The blue solution was used immediately because the monomers dimerized on standing several hours as evidenced by color discharge over this period. The dimer of MNP was soluble in peanut oil and dissociated rapidly to the blue monomer in solution. However, CP was insoluble in peanut oil; therefore, the technique described above for NA in peanut oil was used to prepare an injectable solution which was used immediately because CP precipitated on standing.

Blood AcH determination. Following administration of the test compounds at zero time in the vehicle and the doses listed in Table 1, ethanol (2.0 g/kg, i.p.) was administered at 2 hr. Blood AcH levels were then measured 1 hr after ethanol in treated and control animals as previously described [15].

RESULTS

Inhibition of yeast AlDH in vitro *by C-nitroso compounds.* When incubated with yeast AlDH *in vitro* the aromatic C-nitroso compound NB inhibited this enzyme in a concentration-dependent manner (Fig. 2), as indicated by the failure of the enzyme to oxidize benzaldehyde subsequent to this preincubation period with NB compared to the control enzyme without NB. The IC_{50} for this inhibition was calculated to be 2.5 μM.

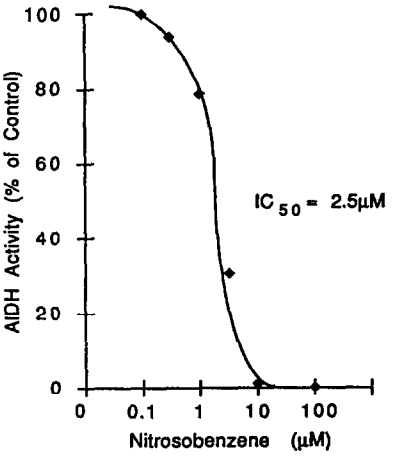


Fig. 2. Concentration–response curve for the inhibition of yeast AlDH by NB. Each point represents the mean of duplicate determinations. The NB was dissolved in DMSO and the assay procedure is described under Methods. The activity of the control was 27.5 ± 0.3 nmol NAD^+ reduced/min. IC_{50} = 50% inhibitory concentration [16].

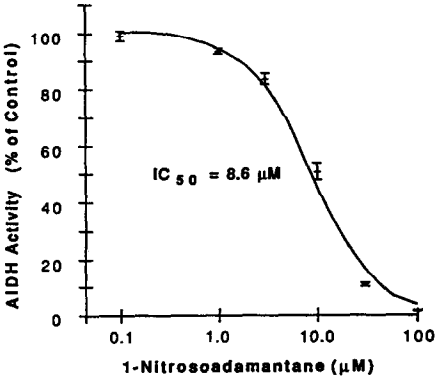


Fig. 3. Concentration–response relationship for the inhibition of yeast AlDH by NA. The NA was dissolved in tetrahydrofuran. Each point is the mean ± SEM of triplicate determinations. The activity of the control was 21.3 ± 0.4 nmol NAD^+ reduced/min. See the legend of Fig. 2 for further details.

NA, an aliphatic C-nitroso compound, similarly inhibited yeast AlDH with an IC_{50} of 8.6 μM (Fig. 3). On the other hand, MNP, the other aliphatic C-nitroso compound (Fig. 1), was considerably less effective (IC_{50} = 0.15 mM) and, at a concentration

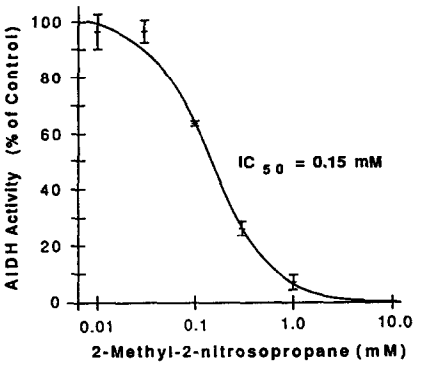


Fig. 4. Concentration–response relationship for the inhibition of yeast AlDH by MNP. MNP was dissolved in DMSO. The data are the means ± SEM of triplicate determinations. The activity of the control was 14.4 ± 0.5 nmol NAD^+ reduced/min. See the legend of Fig. 2 for further details.

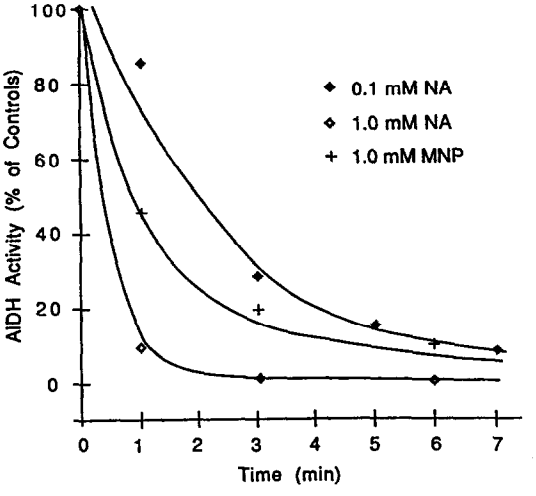


Fig. 5. Time courses for the inhibition of yeast AlDH by NA and MNP. The experimental details are described under Methods. See the legends of Figs. 3 and 4 for further details. Data shown are representative of a typical experiment.

of 100 μM, the enzyme was inhibited by only 36% (Fig. 4). We were unable to compare the activities of the monomeric versus the dimeric forms of NA because of the high reactivity of the monomer; however, it was clear that the activity of a dimethyl sulfoxide (DMSO) solution of MNP increased over time as more MNP dissociated to the monomer (data not shown). The corresponding nitro compounds, viz. 1-nitroadamantane, at 100 μM inhibited the enzyme only 12% (88.3 ± 7.4% of control, $P > 0.05$) under the same conditions, while 2-methyl-2-nitropropane was not inhibitory at 1.0 mM (data not shown). The time courses of inhibition of yeast AlDH by both NA and MNP are shown in Fig. 5. Although the two upper curves appear similar, it should be noted that here the concentrations of NA and MNP differ by an order of magnitude.

In vivo *experiments.* As alluded to earlier, C-nitroso compounds tend to dimerize to complexes that are highly insoluble in aqueous systems because of their symmetry and increased molecular weights. Since the dimeric forms lack the —N=O bond found in nitroxyl (which is presumably required for inhibition of AlDH), and, therefore, would not be expected to inhibit this enzyme (*vide supra*), it was necessary to prepare dosage forms of these C-nitroso compounds with maximal concentrations of the monomer in the injection vehicle. That the dimeric forms might be inactive was indicated by the lack of effect of NA (dimer) in elevating ethanol-derived blood AcH levels when administered (1.0 mmol/kg, i.p.) as a suspension in 2% CMC (Table 1). Because of these difficulties, it was not possible to formulate aqueous dosage forms of these compounds suitable for i.v. administration.

Peanut oil was a vehicle in which these C-nitroso compounds could be solubilized. However, neither NA nor MNP, which turned blue when dissolved in this vehicle, was effective in elevating blood AcH when administered to rats (Table 1). As indicated by the degrees of elevation of blood AcH elicited by CP, the drug used as the control, aqueous CMC appeared to be superior to peanut oil as a drug delivery vehicle, presumably because of the water miscibility of the former. Solutions of NA and MNP in DMSO elicited toxic deaths when administered to rats (data not shown), possibly due to the rapid metabolic formation of dimethyl sulfide whose odor permeated the cages of the animals on this regimen. This unexpected synergistic toxicity of DMSO with C-nitroso compounds is being investigated separately.

DISCUSSION

Metabolically derived nitroxyl, produced by the action of catalase on the alcohol deterrent agent cyanamide, has been postulated to be the putative *in vivo* inhibitor of AlDH, based on deductive evidence and on tracer studies with the ^{15}N- and ^{13}C-labeled cyanamide [1, 2]. More recently, we have shown that prodrugs of nitroxyl that liberate this active species by esterase action on the prodrugs are powerful inhibitors of yeast AlDH *in vitro* [17]. Other prodrugs of nitroxyl that release nitroxyl *in vivo* by the action of the hepatic cytochrome P450 enzymes have been shown to elevate ethanol-derived blood AcH in rats, a consequence of the inhibition of hepatic AlDH [18]. Thus, the evidence that metabolically generated nitroxyl is a good inhibitor of AlDH appears to be firmly established.

Nevertheless, direct proof of nitroxyl involvement in inhibiting AlDH at the molecular level must be obtained by tracing the fate of labeled nitroxyl to the active site of the enzyme itself. Such studies are still pending, but an attractive alternative that may give further credence to this hypothesis is to demonstrate that *substituted* nitroxyls represented by R—N=O, viz. C-nitroso compounds, in fact also inhibit AlDH. That these C-nitroso compounds

Table 1. Elevation of ethanol-derived blood AcH in rats by C-nitroso compounds

Compound	Highest test dose in vehicle (N = 3) (mmol/kg)	Blood AcH (μM) CMC*	Peanut oil*
NB	0.25†	118.0	
NA	1.0	0.0‡	
NA	0.5		0.0
MNP	0.5		0.0
CP control	1.0	215.8 ± 37§	93.6 ± 15§

See Methods for the experimental protocols.
* These vehicles administered alone did not raise ethanol-derived blood AcH.
† N = 1. Lone survivor. NB produced two toxic deaths out of three at this dose.
‡ The NA was suspended in this vehicle as its colorless dimeric form.
§ Mean ± SEM, N = 3.

shown in Fig. 1 are reactive and may therefore interact directly with the enzyme is indicated by their tendency to dimerize (Equation 3) in a manner reminiscent of the dimerization of nitroxyl itself (Equation 2). Accordingly, we evaluated these C-nitroso compounds for their potential to inhibit AlDH.

In accord with our expectations, these C-nitroso compounds turned out to be good inhibitors of yeast AlDH *in vitro* without the need for bioactivation. Both NB and NA exhibited comparable activity, but MNP with the more sterically hindered nitroso group was 20–60 times less active than NA or NB (Figs. 2–5).

However, NA did not show any inhibitory activity on hepatic AlDH *in vivo*, as evidenced by the lack of elevation of blood AcH following ethanol administration to rats (Table 1). This was surprising, since compounds that exhibit strong inhibitory activity toward yeast AlDH *in vitro* almost invariably raise blood AcH when administered to rats [19], a consequence of the inhibition of the hepatic mitochondrial AlDH [20, 21]. While the dimeric form of NA was expected to be inactive, even the monomeric form of NA dissolved in peanut oil did not elicit the expected elevation of ethanol-derived blood AcH when administered to rats in this vehicle. This raises the question as to whether NA ever reached the liver in monomeric form. Thus, lack of specific delivery to the liver as the active monomer appears to be the reason for the absence of any *in vivo* activity by NA. In contrast, NB, which is mostly monomeric, inhibited hepatic AlDH *in vivo* at one-fourth the dose of NA, as reflected by the elevation of blood AcH in the lone surviving rat (Table 1). However, NB was toxic even at this lower dose.

In summary, we have demonstrated that C-nitroso compounds are direct inhibitors of yeast AlDH *in vitro* analogous to the action of nitroxyl itself. These data provide further evidence that nitroxyl-generating compounds and/or nitroxyl-mimics are good inhibitors of AlDH and, when specifically targeted to the liver, may find utility as third generation alcohol deterrent agents for the treatment of alcoholism.

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